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Differential participation of phospholipase A_2 isoforms during iron-induced retinal toxicity. Implications for age-related macular degeneration

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ABSTRACT

Both elevated iron concentrations and the resulting oxidative stress condition are common signs in retinas of patients with age-related macular degeneration (AMD). The role of phospholipase A_2 (PLA_2) during iron-induced retinal toxicity was investigated. To this end, isolated retinas were exposed to increasing Fe^{2+} concentrations (25, 200 or 800 μM) or to the vehicle, and lipid peroxidation levels, mitochondrial function, and the activities of cytosolic PLA_2 ($cPLA_2$) and calcium-independent PLA_2 ($iPLA_2$) were studied. Incubation with Fe^{2+} led to a time- and concentration-dependent increase in retinal lipid peroxidation levels whereas retinal cell viability was only affected after 60 min of oxidative injury.

A differential release of arachidonic acid (AA) and palmitic acid (PAL) catalyzed by $cPLA_2$ and $iPLA_2$ activities, respectively, was also observed in microsomal and cytosolic fractions obtained from retinas incubated with iron. AA release diminished as the association of cyclooxigenase-2 increased in microsomes from retinas exposed to iron. Retinal lipid peroxidation and cell viability were also analyzed in the presence of $cPLA_2$ inhibitor, arachidonoyl trifluoromethyl ketone (ATK), and in the presence of $iPLA_2$ inhibitor, bromoenol lactone (BEL). ATK decreased lipid peroxidation levels and also ERK1/2 activation without affecting cell viability. BEL showed the opposite effect on lipid peroxidation. Our results demonstrate that $iPLA_2$ and $cPLA_2$ are differentially regulated and that they selectively participate in retinal signaling in an experimental model resembling AMD.

1. Introduction

Metallo-neurobiology has undergone a significant evolution in the last 20 years. Still, although there is much experimental evidence on various aspects of the involvement of iron in several neurodegenerative diseases, the role of this metal in the central nervous system and, particularly, in neurodegenerative processes has not been fully elucidated to date (Dunaief, 2006).

Iron is necessary for normal retinal cellular function. Still, iron overload has been found to be associated with retinal degenerative disorders such as ocular siderosis, intraocular hemorrhage, and the hereditary diseases aceruloplasminemia and pantothenate kinase-associated neurodegeneration (Dunaief et al., 2005; Dunaief, 2006; Hadziahmetovic et al., 2008; Hahn et al., 2004; He et al., 2007; Wong et al., 2007). Furthermore, it is well known that reactive oxygen species (ROS) may contribute to the pathogenesis of age-related macular degeneration (AMD) and that they can be produced in the Fenton reaction catalyzed by ferric (Fe^{3+}) and ferrous (Fe^{2+}) ions (Lukinova et al., 2009). Previous post mortem research has found that iron concentrations are higher in AMD retinas than in non-affected retinas (Blasiak et al., 2011). In addition, the age-related increase of iron levels in the macula, the up-regulation of transferrin in AMD, the development of AMD-like

Abbreviations: [^{14}C]DPPC, 1-[^{14}C]palmitoyl-2-[^{14}C]palmitoyl-sn-glycero-3-phosphocholine; [^{14}C]PAPC, 1-palmitoyl-2-[^{14}C]arachidonoyl-sn-glycero-3-phosphocholine; 4-HNE, 4-hydroxynonenal; AA, arachidonic acid; AMD, age-related macular degeneration; ATK, arachidonoyl trifluoromethyl ketone; ATP, adenosine-5′-triphosphate; BEL, bromoenol lactone; BSA, bovine serum albumin; COX, cyclooxigenase; $cPLA_2$, cytosolic phospholipase A_2; DTT, dithiothreitol; EDTA, *N*,*N*′-1,2-ethandiylbis[*N*-(carboxymethyl)glycine] disodium salt; ERK1/2, extracellular signal-regulated kinases; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; HRP, horseradish peroxidase; $iPLA_2$, calcium-independent phospholipase A_2; MAPK, mitogen-activated protein kinases; MEK, mitogen-activated protein kinase kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NBIA, neuronal brain iron accumulation; PAL, palmitic acid; PBS, phosphate buffer saline; PLA_2, phospholipase A_2; PMSF, phenylmethylsulfonyl fluoride; RD, retinal degeneration; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; U0126, 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene.

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syndromes in ceruloplasmin- and hephaestin-deficient mice, and the association between polymorphism of iron homeostasis genes and AMD support the leading role of this metal in this pathology (Blasiak et al., 2011; Garcia-Castineiras, 2010; Hadziahmetovic et al., 2008; Wong et al., 2007). Another disease also characterized by excessive iron accumulation is hemochromatosis. Most patients with hereditary hemochromatosis have a mutation in the histocompatibility leukocyte antigen class I-like protein involved in iron homeostasis (HFE) gene product (Feder et al., 1996). This protein forms, in general, a stable complex with the transferrin receptor, lowering its affinity for transferrin (Feder et al., 1998). Patients with mutations in the HFE gene evidence elevated transferrin binding to transferrin receptors, ending in, in turn, a higher iron uptake into tissues. This genetic disorder is also associated with retinal abnormalities, including, in some cases, retinal pigment epithelium atrophy or angioid streaks. Furthermore, a study conducted in two postmortem patients also showed drusen formation, the clinical hallmark of AMD (Dunaief, 2006).

On the other hand, several neurodegenerative disorders with neuronal brain iron accumulation (NBIA) have been associated with defective phospholipase A_2 (PLA_2) signaling. Mutations in PLA2G6 gene, which encodes a calcium-independent group VI PLA_2, have been reported in NBIA (Morgan et al., 2006). Moreover, the up-regulation of secretory phospholipase A_2 IIA and its participation in neuronal apoptosis have been reported during cerebral ischemia (Adibhatla and Hatcher, 2010; Yagami et al., 2002). PLA_2s belong to a superfamily of enzymes that hydrolyze the sn-2 fatty acids of membrane phospholipids. These proteins are known to play multiple roles related to the maintenance of membrane phospholipid homeostasis and production of a variety of lipid mediators (Burke and Dennis, 2009b). There are more than 20 different types of PLA_2s and, in spite of their common function in hydrolyzing phospholipid fatty acids, they are diversely encoded by a number of genes and are regulated by different mechanisms. The most recent classification involves the following main groups: (i) low molecular secretory PLA_2 ($sPLA_2$) which includes groups I–III, V and IX–XIV; (ii) high molecular calcium-dependent cytosolic PLA_2 ($cPLA_2$) which includes groups IVA–IVF; (iii) high molecular calcium-independent PLA_2 ($iPLA_2$) which includes groups VIA-1, VIA-2 and VIB–VIF; and (iv) platelet-activating factor acetylhydrolase which includes groups VIIA–VIIB and VIIIA–VIIIB (Burke and Dennis, 2009a; Sun et al., 2010).

Although the physiological role of these PLA_2s in the regulation of neuronal cell function has not yet been fully elucidated, there is increasing evidence about their involvement in receptor signaling and transcriptional pathways that link oxidative events to inflammatory responses underlying many neurodegenerative diseases (Sun et al., 2007). Previous research also revealed the important role of $cPLA_2$, $sPLA_2$ and $iPLA_2$ in modulating neuronal excitatory functions, in the inflammatory responses, and, as stated above, in childhood neurological disorders associated with NBIA, respectively (Farooqui et al., 2006; Morgan et al., 2006; Moses et al., 2006; Svensson et al., 2005). Furthermore, the up-regulation of mRNA of various subgroups of $sPLA_2$ during light-induced retinal degeneration (RD) has been reported particularly in the retina (Tanito et al., 2008; Yang et al., 2008). Moreover, the increase in arachidonic acid (AA) release, one of the main products of PLA_2 action, has been suggested to be involved in the pathogenesis of RD (Kashiwagi et al., 2000; Wang and Kolko, 2010). $iPLA_2$ expression has also been involved in the proliferation of retinal pigment epithelium cells during RD associated with AMD (Kolko et al., 2009). Evidence presented here shows a correlation between iron, RD and PLA_2-derived signaling. However, the specific role of PLA_2s in RD still remains unknown. Thus, taking into account this background, the main goal of the present work was to characterize PLA_2 activities and their regulation in an experimental model of RD. To this end, isolated bovine retinas were exposed to increasing Fe^{2+} concentrations, and cellular viability, lipid peroxidation levels and $cPLA_2$ and $iPLA_2$ activities were analyzed. The role of PLA_2 isoforms in retinal damage and the involvement of mitogen-activated protein kinase (MAPK) ERK1/2 in PLA_2 regulation were also studied.

2. Materials and methods

2.1. Materials

1-Palmitoyl-2-[^{14}C]arachidonoyl-sn-glycero-3-phosphocholine (38.0 mCi/mmol, [^{14}C]PAPC) and 1-[^{14}C]palmitoyl-2-[^{14}C]palmitoyl-sn-glycero-3-phosphocholine (111.0 mCi/mmol, [^{14}C]DPPC) were obtained from New England Nuclear-Dupont (Boston, MA, USA). PLA_2 inhibitors [arachidonoyl trifluoromethyl ketone (ATK) and bromoenol lactone (BEL)], mitogen-activated protein kinase kinase (MEK) 1/2 inhibitor [1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene (U0126)], Triton X-100, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), and thiobarbituric acid (TBA) were obtained from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals were of the highest purity available. Mouse polyclonal IgG2a, anti-phospho-Tyr204-extracellular signal-regulated kinases (ERK) 1/2, rabbit polyclonal anti-phosphotyrosine (PY20), rabbit polyclonal anti-ERK2, rabbit polyclonal anti-phospho-Thr180/Tyr182-p38, rabbit polyclonal anti-β-tubulin, rabbit polyclonal anti-calnexin, polyclonal horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG, and polyclonal HRP-conjugated goat anti-mouse IgG were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Rabbit polyclonal anti-cyclooxygenase (COX)-2 was purchased from Cayman Chemical (Ann Arbor, MI, USA). Rabbit anti-phospho-Ser505-$cPLA_2$ was purchased from Cell Signaling Technology (Boston, MA, USA).

2.2. Experimental treatments

Fresh bovine eyes were obtained from a local abattoir and placed and stored in crushed ice. Retinas were dissected on ice (4 °C) under normal lighting conditions and washed with saline solution. Entire retinas were preincubated at 37 °C for 30 min with either inhibitors (50 μM ATK, 25 μM BEL or 10 μM U0126) or the vehicle, and they were subsequently exposed for 5 or 60 min to either $FeSO_4$ (25, 200 or 800 μM) or its vehicle as previously described (Uranga et al., 2007). Entire retinas were incubated under an O_2:CO_2 (95:5, vol/vol) atmosphere with gentle agitation for all experiments. All incubations were performed in Locke's buffer [154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 1 mM $MgCl_2$, 2.3 mM $CaCl_2$, 5 mg/ml glucose, 5 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), pH 7.2] unless stated otherwise. After incubation, retinas were washed in Locke's buffer to be further used for experimental procedures.

2.3. Isolation of subcellular fractions

Subcellular fractions were obtained as previously described with slight modifications (Salvador and Giusto, 2006). Briefly, homogenates (20% wt/vol) from the dissected retinas to be used for subcellular fractionation were prepared in a medium containing 0.32 M sucrose, 1 mM *N*,*N*′-1,2-ethandiylbis[*N*-(carboxymethyl)glycine] disodium salt (EDTA), 1 mM dithiothreitol (DTT), 2 mg/ml leupeptin, 1 mg/ml aprotinin, 1 mg/ml pepstatin, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 mM HEPES (pH 7.4). Homogenates to be used for MTT reduction, thiobarbituric acid reactive substances (TBARS) and Western blot assays, and